AD_____

Award Number: W81XWH-07-1-0563

TITLE: Single-Walled Carbon Nanotubes Targeted to the Tumor Vasculature for Breast Cancer Treatment

PRINCIPAL INVESTIGATOR: Roger G. Harrison

CONTRACTING ORGANIZATION: University of Oklahoma
Norman, OK 73019-0430

REPORT DATE: September 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

Form Approved
OMB No. 0704-0188

REPORT DOCUMENTATION PAGE

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-09-2009	Final	16 Aug 2007 – 15 Aug 2009

4. TITLE AND SUBTITLE
Single-Walled Carbon Nanotubes Targeted to the Tumor Vasculature for Breast Breast Cancer Treatment

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0563

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Roger G. Harrison, Daniel E. Resasco, and Peter S. McFetridge

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Oklahoma

Norman, OK 73019-0430

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This project explores a novel treatment of breast cancer that uses single-walled carbon nanotubes (SWNTs) in photodynamic therapy, in which the SWNTs are targeted to the endothelial cells that line the tumor vasculature. The purpose of this project is to evaluate this treatment concept using human endothelial cells *in vitro.* Recombinant annexin V has been produced in good purity and high yield, and it has been shown to bind strongly to plastic-immobilized phosphatidylserine (PS), with a dissociation constant (K_d) of 5.1 nM. A new method was developed for conjugating amino groups of annexin V to single-walled carbon nanotubes (SWNTs) that retains the strong absorbance of the SWNTs at 980 nm. This method uses the linker fluorenylmethoxycarbonyl (Fmoc)-amine-PEG-succinimidyl carboxy methyl ester. The loading of annexin V on the SWNTs was high (7.7 mg protein/mg SWNTs). Human endothelial cells grown *in vitro* could be killed almost completely when SWNT-Fmoc-PEG-annexin V was bound to the cells and with laser light at 980 nm and an energy density of 195 J/cm^2. By contract, there was no harm to cells with SWNT-Fmoc-PEG-annexin V bound and no laser treatment, and there was no harm to cells with no SWNT-Fmoc-PEG-annexin V bound receiving laser treatment.

15. SUBJECT TERMS
Single-walled carbon nanotubes, annexin V, photodynamic therapy, breast tumor vasculature

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

This project explores a novel treatment of breast cancer that uses single-walled carbon nanotubes (SWNTs) in photodynamic therapy (PDT). SWNTs are unique in that they strongly absorb near-infrared (NIR) light, while biological systems have relatively low levels of absorption of NIR light. In order to have a simple and fast means of delivery of the SWNTs to the tumor, it is proposed to target the SWNTs by conjugation with human annexin V, which has been shown to bind to phosphatidylserine (PS) exposed on the surface of endothelial cells in blood vessels in tumors; PS is not exposed on the surface of endothlelial cells in blood vessels in normal organs. The purpose of this project is to evaluate this treatment concept using human endothelial cells *in vitro* and NIR light at 980 nm.

BODY

The research accomplishments of this project are summarized as follows:

Task 1 – Production of the SWNT-annexin V complex

Recombinant annexin V was produced and purified throughout the project period using the procedures we have used for other recombinant proteins (Zang et al. 2006). Yield and purity data for the annexin V was given in the 2008 annual project report.

Considerable effort was devoted to finding a method to conjugate annexin V to the SWNTs that keeps the SWNTs suspended in aqueous media and also retains the strong absorption of NIR light at 980 nm. The various methods that were tried are summarized as follows:

1. Adsorption to the SWNT surface – We developed this method for the model protein horseradish peroxidase (HRP) using a suspension-dialysis method (Palwai et al. 2007). The SWNTs are suspended using an aqueous solution of sodium cholate using sonication. The protein is added, and the suspension is dialyzed using a membrane that allows sodium cholate to pass through but retains the protein. The suspension is then dialyzed with a membrane that allows the protein to pass through. When this method was applied to annexin V, precipitation of the SWNTs resulted after the dialysis step.

2. Suspension of SWNTs using carboxymethylcellulose (CMC) and covalent attachment of annexin V to the CMC – This method is described in the 2008 annual report; this method uses a 100 kDa dialysis membrane to remove unbound CMC by dialysis. Since then, we have use a 300 kDa membrane for the dialysis, but we found from FTIR measurements that little CMC is removed even using this membrane. Therefore, we have concluded that this method is not feasible because of the low removal of unadsorbed CMC by dialysis.

3. Adsorption of pyrenebutanoyl succinimide followed by reaction with the protein – This method was used by Shao et al. (Shao et al. 2007) to conjugate monoclonal antibodies to SWNTs. In this method, the SWNTs are suspended by sonication in aqueous buffer and

centrifuged to remove aggregates. Pyrenebutanoyl succinimide dissolved in methanol is added to the SWNT suspension. After mixing, polyethylene glycol (PEG) with a molecular weight of 8000 is added to cover unoccupied sites on the SWNTs so that undesirable binding with other biomolecules is prevented. Dialysis is performed with a 100 kDa membrane to remove unadsorbed pyrenebutanoyl succinimide and PEG. Finally, the monoclonal antibody is added, and the succinimide reacts with the epsilon amino groups of lysines or the N-terminal amine of the protein. When this method was tried, the SWNTs precipitated after the centrifugation step. This method was modified by first suspending the SWNTs in sodium cholate, and then pyrenebutanoyl succinimide dissolved in methanol was added. After dialysis with buffer, the SWNTs precipitated. From these experiments, we have concluded that the Shao et al. method for attachment of proteins to SWNTs, or variations of it, will not work for the SWNTs we are using that are rich in the (6,5) type and are produced by the CoMoCAT method. For the tests of treatment of endothelial cells with SWNTs bound (see Task 3), we are using a laser with a wavelength of 980 nm, so we need to use SWNTs with a peak in absorbance at 980 nm. The CoMoCAT (6,5) type SWNTs we are using have a peak in absorbance at 980 nm, while the SWNTs that Shao et al. used have a peak at 808 nm.

4. Adsorption of a linker containing fluorene followed by reaction with the protein – This is a new method we developed that uses a linker that contains a fluorene aromatic group for strong π-π binding to the graphite nanotube sidewall, polyethylene glycol (PEG) to increase the aqueous solubility of the linker, and a succinimide ester group for covalent bonding with the ε-amino group of lysines or the amino-terminal group of the protein to give a stable amide linkage. The linker is shown in Figure 1, and a schematic representation of the protein attachment via the linker to the SWNT wall is shown in Figure 2.

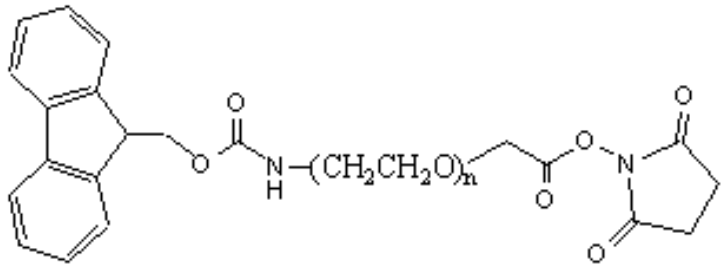


Figure 1. Fmoc-amine-PEG-succinimidyl carboxy methyl ester. Fmoc: fluorenylmethoxycarbonyl.

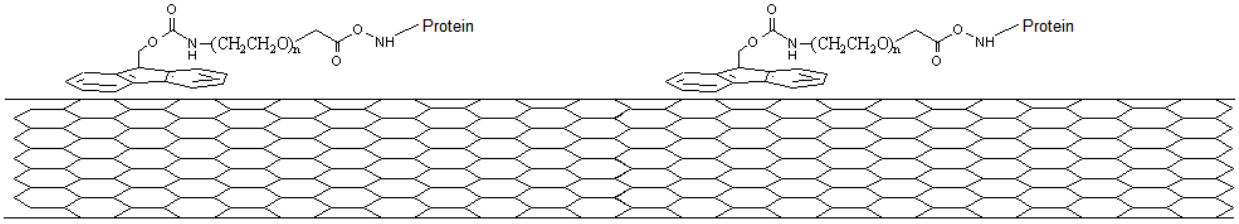


Figure 2. Schematic of protein attachment to an SWNT using the Fmoc-amine-PEG-succinimidyl carboxy methyl ester linker.

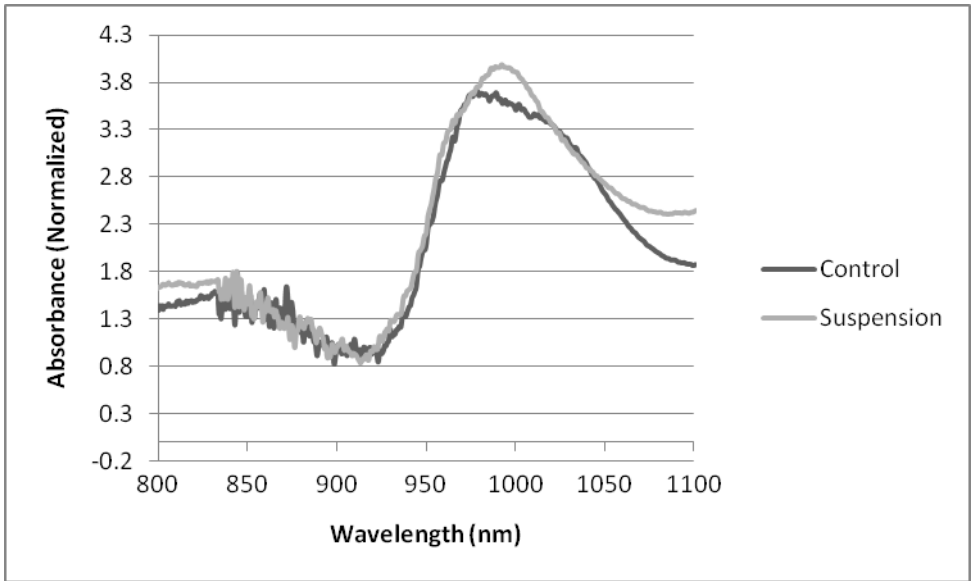
We have used fluorene in the linker because it is well known that condensed aromatic rings can be used as anchor groups on carbon surfaces (Katz 1994). Fluorene has been shown to bind strongly to graphite (Kalashnikova et al. 1981), and it is believed that this strong binding is facilitated by the orientation of the fluorene molecule to enable π-π interactions.

The first step in this method is to completely suspend the SWNTs in an aqueous solution of sodium cholate using sonication. After centrifugation, an aqueous solution of the linker is added, and the suspension is mixed. Annexin V is then added in an equimolar quantity and allowed to react with the succinimide ester group of the linker. The suspension is dialyzed using a 100 kDa dialysis membrane, which will allow any unreacted protein and sodium cholate to pass through. After centrifugation, this suspension had a protein concentration of 40 mg/liter. This suspension was used in laser tests with endothelial cells and resulted in almost killing of the endothelial cells (see Task 3).

The method, as just described, was not able to be routinely reproduced. To investigate the problem, we substituted horseradish peroxidase (HRP) for annexin V and added varying amounts of bovine serum albumin (BSA) after the addition of HRP to the SWNT suspension. We added BSA to stabilize the SWNTs and keep them from aggregating. As the amount of BSA was decreased by a factor of 94, the SWNT concentration fell by a factor of 13, while the HRP enzyme activity dropped by only 20%. From this result, we have concluded that the HRP activity was only slightly affected by the reaction to succinimide but that HRP was not associated with the SWNTs. Instead, the data are consistent with the self-aggregation of the fluorene groups of the linker. A self-aggregated linker would react with two HRP molecules, and this complex (MW = 88 kDa) would not readily pass through the 100 kDa dialysis membrane.

To overcome the problem of the linker being self-aggregated, we modified the procedure so that binding of the linker to the SWNT surface is favored. For this procedure, we substituted sodium dodecylsulfate (SDS) for sodium cholate. SDS adsorbs less strongly to SWNTs than sodium cholate (Wenseleers et al. 2004). The SWNTs are suspended in an aqueous solution of SDS using sonication. After centrifugation, an aqueous solution

of the linker with the same concentration of SDS is added to the SWNT suspension. Dialysis using a 2 kDa membrane is performed to remove SDS (MW = 0.28 kDa) but retain the linker (MW = 3.78 kDa). The suspension is centrifuged, and an equimolar amount of annexin V is added. Finally, dialysis is performed with a 100 kDa membrane to remove any unreacted protein, and the suspension is centrifuged. This procedure resulted in an annexin V concentration of 783 mg/liter and a SWNT concentration of 102 mg/liter. The NIR spectra of the suspension before and after addition of the protein showed that the absorbance peak at 980 nm was completely retained (Figure 3). There was a slight red shift of ~10 nm in the peak, which we have seen previously when protein was adsorbed on the SWNT surface (Palwai et al. 2007) and has also been seen by others for adsorbed DNA (Malik et al. 2007). It is interesting to note that when we suspended the SWNTs by adsorbing the protein horseradish peroxidase, the absorption peak at 980 nm was about 60% of the peak when the SWNTs were suspended using the surfactant sodium cholate (Palwai et al. 2007). Thus, it is likely that annexin V is not adsorbing to the SWNT surface when the Fmoc-amine-PEG-succinimidyl carboxy methyl ester linker is used in the suspension procedure. The complete experimental protocol is given in Appendix I.



0

Figure 3. Absorption spectra of SWNTs with annexin V attached via the Fmoc-amine-PEG-succinimidyl carboxy methyl ester linker (suspension) and suspended using SDS (control).

Task 2 – Test of the function of the SWNT-annexin V complex

In the 2008 annual report, data was given for the binding of annexin V to PS immobilized on plastic microtiter plates and for the binding of the SWNT-CMC-annexin V complex to human endothelial cells in which PS was exposed on the surface of the cells by the addition of a low

level of hydrogen peroxide (1 mM). The protocols for these binding assays were given in the 2008 annual report. The dissociation constant (K_d) was found to be 5.1 nM and 2.9 nM for binding to PS on microtiter plates and on the surface of endothelial cells, respectively, indicating relatively strong binding. From our work on the conjugation of annexin V to CMC adsorbed to SWNTs (see Task 1) and on a laser test of cells that had been exposed to SWNT-CMC-annexin V (see Task 3), we know that it is very likely that we actually were measuring the binding of CMC-annexin V to PS on the cells. This shows that annexin V is still functional after primary amino groups of annexin V react with carboxy groups of CMC.

Task 3 – Test of the effect of NIR light on endothelial cells with SWNT-annexin V bound

Data was given in the 2008 annual report on a test to determine the laser energy density (J/cm^2) that could be used without harming normal cells. The result was that the laser light at 980 nm does not significantly affect the ability of the cells to grow at or below 360 J/cm^2. Data was also given in the 2008 annual report on the effect of laser light at 980 nm on human endothelial cells grown *in vitro* with and without the addition of SWNT-CMC-annexin V complex. A test to measure the metabolic activity of the cells using Alamar Blue did not indicate a conclusive effect of the combination of laser light and the SWNT-CMC-annexin V complex. Cells with and without the presence of the SWNT-CMC-annexin V complex were observed by a microscope, and no differences were observed (i.e. the cells in the presence of the SWNT-CMC-annexin V complex did not appear darker because of the black SWNTs bound). These findings indicate that annexin V was conjugated mainly to CMC.

SWNTs with annexin V conjugated using the Fmoc-PEG-succinimide linker were used in a laser test with endothelial cells. This conjugate had a protein concentration of 40 mg/liter at the end of the preparation procedure (see Task 1). The same procedure as given in the 2008 annual report was used (SWNT-annexin V complex of 20 nM protein and energy density of about 200 J/cm^2). The results are shown in Figure 4 and indicate that the cells with SWNT-Fmoc-PEG-annexin V bound were killed almost completely with laser light at 980 nm and an energy density of 195 J/cm^2, with no harm to cells with SWNT-Fmoc-PEG-annexin V bound and no laser treatment and with no harm to cells with no SWNT-Fmoc-PEG-annexin V bound receiving laser treatment. Observations of the cells using a microscope were consistent with the results shown in Figure 4. Experiments are underway to repeat this test using SWNTs conjugated with annexin V using the same linker but using SDS in the conjugation procedure and an additional dialysis using a 2 kDa membrane (see Task 1 and the protocol in Appendix I). After these experiments are successfully concluded, an article describing this research will be submitted to a journal for publication. The target date for this submission is November 1.

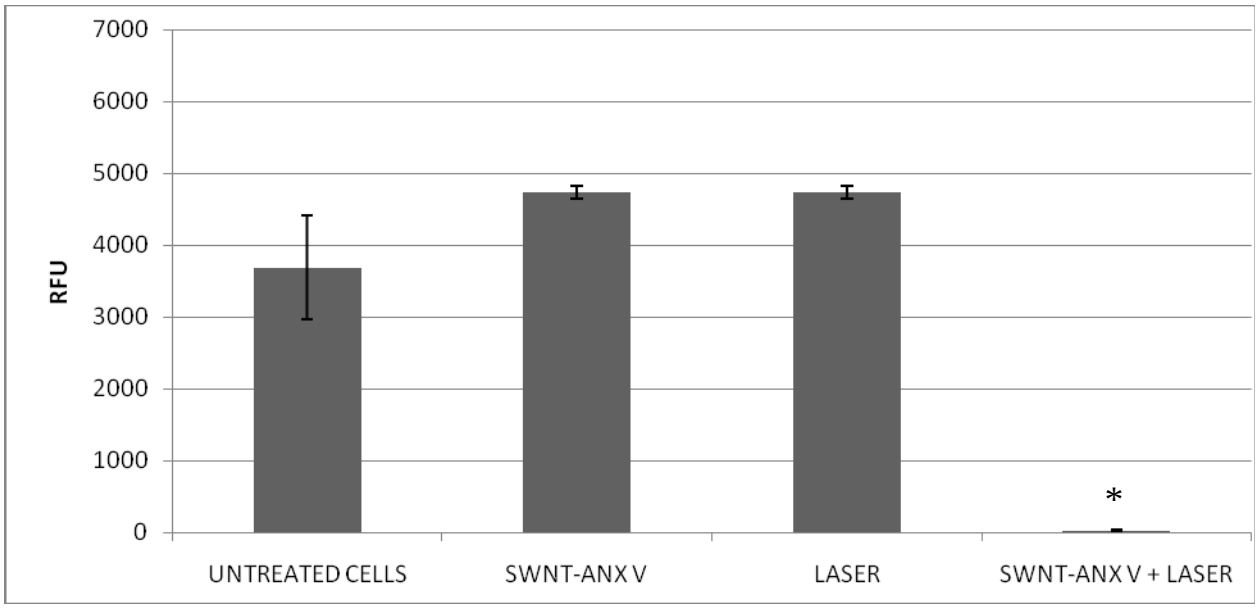


Figure 4. Effect of laser light at 980 nm on human endothelial cells grown in 24-well plates. For cells treated with the laser, the power was 1.5 W/cm^2 for 130 sec (195 J/cm^2). For cells treated with the SWNT-Fmoc-PEG-annexin V complex, the annexin V concentration in the wells was 20 nM. The (*) symbol indicates that RFU is significantly different compared to untreated cells ($p < 0.05$). The bars indicate S.E.

KEY RESEARCH ACCOMPLISHMENTS

- Purified recombinant annexin V was produced in good purity (> 99%) and high yield (145 mg/liter of starging culture broth).
- A new method was developed for conjugating annexin V to SWNTs that retains the strong absorbance of the SWNTs at 980 nm. This method uses the linker fluorenylmethoxycarbonyl (Fmoc)-amine-PEG-succinimidyl carboxy methyl ester. The SWNTs stayed suspended after the addition of this linker and after the surfactant (SDS) originally used to suspend the SWNTs was removed. This procedure resulted in a high loading of annexin V on the SWNTs (7.7 mg protein/mg SWNTs).
- The purified annexin V strongly binds to plastic-immobilized PS as indicated by its dissociation constant (5.1 nM). The purified annexin V retains its strong binding to PS on human endothelial cells after it is conjugated to CMC, as indicated by its dissociation constant (2.9 nM). This shows that annexin V is still functional after primary amino groups of annexin V react with carboxy groups of CMC.
- The energy density range that can be used for treatment of human endothelial cells *in vitro* without significantly affecting the ability of the cells to grow was determined (up to 360 J/cm^2).
- Human endothelial cells grown *in vitro* could be killed almost completely when SWNT-Fmoc-PEG-annexin V was bound to the cells and with laser light at 980 nm and an

energy density of 195 J/cm^2. By contract, there was no harm to cells with SWNT-Fmoc-PEG-annexin V bound and no laser treatment, and there was no harm to cells with no SWNT-Fmoc-PEG-annexin V bound receiving laser treatment. These results provide an excellent basis for future testing of this test in mice with implanted tumors.

REPORTABLE OUTCOMES (Abstracts of meeting presentations are given in Appendix II.)

Harrison, R.G., and Resasco, D.E., "Composition and Method for Cancer Treatment Using Targeted Single-Walled Carbon Nanotubes," U.S. Patent Application, February 19, 2008.

Palwai, N.R., Martyn, D.E., Resasco, D.E., Neves, L.F.F., Tan, Y., and Harrison, R.G. (speaker), "Attachment of Proteins to Single-Walled Carbon Nanotubes for Targeting and Treatment of Tumors," AIChE Annual Meeting, Salt Lake City, November, 2007.

Harrison, R.G. (invited speaker), "Novel Fusion Proteins Designed for Targeted Therapies," Cambridge Healthtech Institute's Fourth Annual Conference on Peptide and Protein-Based Therapeutics, San Diego, CA, January 7, 2008.

Neves, L.F.F., Lazrak, Y., Tran, K., Martyn, D.E., Resasco, D.E., and Harrison, R.G., "Endothelial Cell Binding Properties and Absorption Spectra of Single-Walled Carbon Nanotubes with Human Protein Annexin V Attached," poster presentation at the 37th Annual Biochemical Engineering Symposium, Iowa State University, Ames, Iowa, April 26, 2008.

Neves, L.F.F., Lazrak, Y., Martyn, D.E., Resasco, D.E., and Harrison, R.G. (presenter), "Single-Walled Carbon Nanotubes Targeted to the Tumor Vasculature for Breast Cancer Treatment," poster presentation, Department of Defense Era of Hope Breast Cancer Research Meeting, Baltimore, June 26, 2008.

Neves, L.F.F., Lazrak, Y.A., Martyn, D.E., McFetridge, P.S., Resasco, D.E., and Harrison, R.G., "Novel Therapy for Cancer Using Single-Walled Carbon Nanotubes" Biomedical Engineering Society Annual Meeting, St. Louis, October, 2008.

Harrison, R.G. (speaker), Neves, L.F.F., Lazrak, Y.A., Martyn, D.E., McFetridge, P.S., Resasco, D.E., and Bartels, K.E., "Targeting Single-Walled Carbon Nanotubes for the Treatment of Cancer," AIChE Annual Meeting, Philadelphia, November, 2008.

Neves, L.F.F., Tsai, T.W., Palwai, N.R., Martyn, D.E., Tan, Y., Schmidtke, D.W., Resasco, D.E., and Harrison, R.G.," Non-Covalent Attachment of Proteins to Single-Walled Carbon Nanotubes," In: Methods in Molecular Biology: Carbon Nanotubes, M. Burghard and K. Balasubramanian (Eds.), Humana Press, in press.

"Carbon Nanotube Technology Center (CANTEC)," DOE, D.E. Resasco, PI, R.G. Harrison, Co-PI, and five additional Co-PI's, 8-1-08 to 7-31-10, $960,000 (Title of project directed by R.G. Harrison: "Targeted Single-Walled Carbon Nanotubes in Photodynamic Therapy for Cancer." The objective of this project is to study the effect of the SWNT-annexin V complex on an

orthotopic human breast tumor xenograft in mice using i.v. injection followed by exposure of the xenograft to NIR light. There is no overlap of this project with the DOD Breast Cancer Research Concept Award, since the DOD award is for work only with cells *in vitro.*).

CONCLUSION

Recombinant annexin V has been produced in good purity and high yield, and it has been shown to bind strongly to plastic-immobilized PS (dissociation constant of 5.1 nM). A binding assay was performed with the CMC-annexin V conjugate; this conjugation involves the reaction of carboxyl groups of CMC with amino groups of the protein. A binding assay of this conjugate to human endothelial cells with PS exposed on the cell surface showed a strong binding (dissociation constant of 2.9 nM). This shows that annexin V is still functional after primary amino groups of annexin V react with carboxy groups of CMC. A new method was developed for conjugating amino groups of annexin V (ε- amino group of lysines or the amino-terminal group of the protein) to SWNTs that retains the strong absorbance of the SWNTs at 980 nm. This method uses the linker fluorenylmethoxycarbonyl (Fmoc)-amine-PEG-succinimidyl carboxy methyl ester. The SWNTs stayed suspended after the addition of this linker and after the surfactant (SDS) used to suspend the SWNTs originally was removed. The loading of annexin V on the SWNTs was high (7.7 mg protein/mg SWNTs). Human endothelial cells grown *in vitro* could be killed almost completely when SWNT-Fmoc-PEG-annexin V was bound to the cells and with laser light at 980 nm and an energy density of 195 J/cm^2. By contract, there was no harm to cells with SWNT-Fmoc-PEG-annexin V bound and no laser treatment, and there was no harm to cells with no SWNT-Fmoc-PEG-annexin V bound receiving laser treatment.

The results of this project provide an excellent basis for future testing of this test in mice with implanted tumors. Future work is planned to demonstrate this concept using whole animal studies in animals with a fully functioning immune system. Using animals with a complete immune system is a more realistic test of this concept that the commonly used nude mouse models. The mouse model that uses 4T1 mouse breast tumors implanted in female BALB/c mice will be tested

This DOD Breast Cancer Research Program Concept project has the potential for a revolutionary impact on the treatment of breast cancer. There would be significantly fewer side effects than for conventional PDT, because the light-sensitive agent (SWNTs) is targeted specifically to the tumor. This therapy would be much less invasive than surgery and could replace surgery in some cases, which would result in much less pain and disfigurement to breast cancer patients and much shorter stays in the hospital.

REFERENCES

Kalashnikova EV, Kiselev AV, K.D. S, Vasileva SD. 1981. Retention of diphenyls, terphenyls, phenylalkanes and fluorene on graphitized thermal carbon black. Chromatographia 14:510-514.

Katz E. 1994. Application of bifunctional reagents for immobilization of proteins on a carbon electrode surface: oriented immobilization of photosynthetic reaction centers. J Electroanal Chem 365:157-164.

Malik S, Vogel S, Rosner H, Arnold K, Hennrich F, Kohler AK, Richert C, Kappes MM. 2007. Physical chemical characterization of DNA-SWNT suspensions and associated composites. Composites Science and Technology 67:916-921.

Palwai NR, Martyn DE, Neves LFF, Tan Y, Resasco DE, Harrison RG. 2007. Retention of biological activity and near-infrared absorbance upon adsorption of horseradish peroxidase on single-walled carbon nanotubes. Nanotechnology 18:235601.

Shao N, Lu S, Wickstrom E, Panchapakesan B. 2007. Integrated molecular targeting of IGF1R and HER2 surface receptors and destruction of breast cancer cells using single wall carbon nanotubes. Nanotechnology 18:315101.

Wenseleers W, Vasov II, Goovaerts E, Obraztsova ED, Lobach AS, A. B. 2004. Efficient isolation and soubilization of pristine single-walled nanotubes in bile salt micelles. Adv Funct Mater 14:1105-1112.

Zang XP, Palwai NR, Lerner MR, Brackett DJ, Pento JT, Harrison RG. 2006. Targeting a methioninase-containing fusion protein to breast cancer urokinase receptors inhibits growth and migration. Anticancer Res 26:1745-1751.

APPENDIX I

Procedure for the Conjugation of Annexin V to SWNTs Using the Linker Fluorenylmethoxycarbonyl (Fmoc)-amine-PEG-succinimidyl Carboxy Methyl Ester

1. Add 3 mg of SWNTs to 7 ml of a 1 % sodium dodecylsulfate (SDS) solution.
2. Sonicate the suspension for 30 min.
3. Centrifuge the suspension for 30 min at 29,600 x g to remove any aggregated SWNTs.
4. Dissolve 1 mg of Fmoc-NH-PEG-SCM (Creative PEGWorks, Winston Salem, NC) in 1 ml of the 1 % SDS solution.
5. Dissolve 4 mg of the protein annexin V in 4 ml of 40 mM sodium phosphate buffer.
6. Add 400 µl of the linker solution to half the nanotube suspension (3.5 ml) and mix for 30 min.
7. Perform a 24 h dialysis using a 2 kDa dialysis membrane. Change the buffer after 4, 8, 20 h from the beginning of the dialysis. The buffer to be used in this step is 20 mM sodium phosphate buffer at pH 7.4. The volume of the buffer to be used is 1 L. The last dialysis step is performed for 4 h. (Volume to be dialysed = 3.9 ml).
8. Perform a 1 h centrifugation at 29,600 x g in order to remove any SWNTs aggregates.
9. Add 3.7 ml of the protein solution to the centrifuged suspension and allow it to mix with the SWNT suspension for 30 min (equal molar ratio to the linker).
10. Perform a 24 h dialysis using a 100 kDa dialysis membrane. Change the buffer after 4, 8, 20 h from the beginning of the dialysis. The buffer to be used in this step is 20 mM sodium phosphate buffer at pH 7.4. The volume of the buffer to be used is 1.5 L. The last dialysis step is performed for 4 h. (Volume to be dialysed = 7.6 ml).
11. Perform a 1 h centrifugation at 29,600 x g in order to remove any SWNTs aggregates.
12. After centrifugation, measure the SWNT and the protein concentrations.

APPENDIX II

Abstracts of Meeting Presentations

1. Palwai, N.R., Martyn, D.E., Resasco, D.E., Neves, L.F.F., Tan, Y., and Harrison, R.G. (speaker), "Attachment of Proteins to Single-Walled Carbon Nanotubes for Targeting and Treatment of Tumors," AIChE Annual Meeting, Salt Lake City, November, 2007.

Single-walled carbon nanotubes (SWNTs) are unique in that they strongly absorb near-infrared (NIR) light, while biological systems have very low levels of absorption of NIR light. It has recently been shown that cancer cells could internalize SWNTs functionalized with a folate moiety attached and that the cells could be then be killed by exposure of the cells to NIR radiation with an 808 nm laser, with cell death caused by extensive local heating of SWNTs inside the cells. For cancer treatment, this will require that the SWNTs be delivered directly to the cancer cell surface.

To avoid the difficult problem of delivering the SWNTs to the cancer cell surface, this project focuses on developing a means for targeting the SWNTs to the tumor vasculature. It is proposed to target the SWNTs by conjugation with the human annexin V protein. Human annexin V (molecular weight of 36,000) is a monomeric protein that binds with high affinity to phophatidylserine (PS) in phospholipid bilayers. PS is the most abundant anionic phospholipid of the plasma membrane and is tightly segregated to the internal side of the plasma membrane in most cell types. Anionic phospholipids are largely absent from the external surfaces of resting mammalian cells under normal conditions. Recently, it has been found that PS is expressed on the external surface of endothelial cells that line the blood vessels in tumors but is not expressed on the outside surface of the vascular endothelium in normal organs. Thus, annexin V can be used to specifically target the endothelial cells of the tumor vasculature. The underlying hypothesis is that a SWNT-annexin V conjugate injected into the bloodstream will localize in the tumor vasculature and that exposure of the tumor to NIR radiation will cause the tumor to be selectively killed by a combination of heating and cutting off its blood supply.

The first objective of this project is to demonstrate protein attachment to fully suspended SWNTs with no loss of protein activity or loss of the NIR absorption properties of the SWNTs. This work was first carried out with the model protein horseradish peroxidase (HRP), which has a size similar to that of annexin V (40 kDa) and is also a monomeric protein. Two approaches were used for attachment of HRP: (1) adsorption on SWNTs using the sodium cholate suspension-dialysis method, and (2) covalent conjugation to carboxymethylcellulose (CMC) adsorbed to the SNWTs. Both of these methods gave good suspension of SWNTs and almost complete retention of native HRP enzymatic activity before attachment. Both methods also resulted in retention of a substantial fraction of the NIR absorption peak at 980 nm. Atomic force microscopy (AFM) images of the SWNT/protein complexes were obtained.

The sodium cholate suspension-dialysis method was applied to purified recombinant annexin V produced in our laboratory for adsorption on SWNTs. For this protein, the method resulted in complete precipitation of the SNWT/protein complex. Therefore, annexin V was

covalently coupled to CMC adsorbed to the SWNTs. The results of an *in vitro* binding assay of SWMT/CMC/annexin V to PS will be discussed.

2. Harrison, R.G. (invited speaker), "Novel Fusion Proteins Designed for Targeted Therapies," Cambridge Healthtech Institute's Fourth Annual Conference on Peptide and Protein-Based Therapeutics, San Diego, CA, January 7, 2008.

Basic research over the past two decades has enabled the identification of target molecules that have the potential for being used in new therapies that are targeted and have few or no side effects. We have developed several novel fusion proteins with potential therapeutic applications that are designed based on molecular targeting. A consideration in the design of these fusion protein is minimizing problems with delivery. Fusion proteins, either alone or conjugated to single-walled carbon nanotubes, will be discussed that are designed to treat solid tumors or bleeding disorders.

3. Neves, L.F.F., Lazrak, Y., Tran, K., Martyn, D.E., Resasco, D.E., and Harrison, R.G., "Endothelial Cell Binding Properties and Absorption Spectra of Single-Walled Carbon Nanotubes with Human Protein Annexin V Attached," poster presentation at the 37th Annual Biochemical Engineering Symposium, Iowa State University, Ames, Iowa, April 26, 2008.

To develop a therapeutic system with cancer cell selectivity, the present study proposes a possible specific and localized tumoral treatment. Phosphatidylserine exposure on the external face of the cell membrane is almost completely exclusive to cancer cells and endothelial cells in the tumor vasculature. With this knowledge and because of the fact that the protein annexin V has excellent Ca^{2+}-phospholipid binding properties, we have excellent conditions for the development of a therapeutic system.

In this study, we have evaluated the binding of the protein annexin V to single-walled carbon nanotubes (SWNTs) by using a zero-length crosslinking agent that couples carboxyl groups to primary amines (EDC). The binding of recombinant annexin V to phosphatidyl serine immobilized on plastic propylene microtiter plates and on the surface of cancer cells was evaluated by ELISA. The results had shown a correlation between the annexin V concentration and the strength of the binding to the phosphatidyl serine.

4. Neves, L.F.F., Lazrak, Y., Martyn, D.E., Resasco, D.E., and Harrison, R.G. (presenter), "Single-Walled Carbon Nanotubes Targeted to the Tumor Vasculature for Breast Cancer Treatment," poster presentation, Department of Defense Era of Hope Breast Cancer Research Meeting, Baltimore, June 26, 2008.

Background and objectives: This project explores a novel treatment of breast cancer that uses single-walled carbon nanotubes (SWNTs) in photodynamic therapy (PDT). SWNTs are unique in that they strongly absorb near-infrared (NIR) light, while biological systems have very low levels of absorption of NIR light. In order to have a simple means of delivery of SWNTs to

the tumor, it is proposed to target the SWNTs by conjugation with human annexin V. SWNTs that absorb NIR light strongly at 980 nm will be used, which penetrate more deeply in tissue than NIR light at lower wavelengths. Human annexin V is a protein that binds with high affinity to phophatidylserine (PS) in phospholipid bilayers. PS is the most abundant anionic phospholipid of the plasma membrane and is tightly segregated to the internal side of the plasma membrane in most cell types. Recently, it has been found that PS is expressed on the external surface of endothelial cells that line the blood vessels in tumors but is not expressed on the outside surface of the vascular endothelium in normal organs. Thus, annexin V can be used to specifically target the endothelial cells of the tumor vasculature. The objectives of this project are to (1) produce by recombinant DNA technology human annexin V and conjugate the annexin V to SWNTs), (2) verify that the SWNT-annexin V complex will bind specifically to surface-immobilized PS *in vitro* and to the surface of human endothelial cells *in vitro* in which PS has been induced to be on the cell surface, and (3) demonstrate that endothelial cells with SWNT-annexin V bound can be killed by NIR light.

Methods: Annexin V will be produced by recombinant *Escherichia coli* and then purified to homogeneity. SWNTs will be dispersed in the presence of carboxymethylcellulose (CMC) to give a SWNT-CMC complex. Annexin V will be covalently coupled to CMC on the SWNTs using 1-ethyl-3-[dimethylaminopropyl]carbodiimide hydrochloride (EDC). The binding of SWNT-annexin V to PS will be measured using PS immobilized on plastic microtiter plates and also using human endothelial cells *in vitro* in which PS has been induced to be on the cell surface by the addition of a low concentration of hydrogen peroxide. The cells with SWNT-annexin V bound will be exposed to NIR light at 980 nm for various times and power levels. At the end of each period of exposure to NIR light, cell viability will be determined.

Results to date: Annexin V has been produced in *E. coli* and purified to homogeneity. The amino-terminal sequence (first six amino acids) of the protein has been found to be correct. Annexin V has been covalently coupled to SWNT-CMC using EDC, and the SWNT-CMC-annexin V complex has been found to stay stably suspended. Human endothelial cells have been obtained from the ATCC and successfully grown in the laboratory; aliquots of cells have been frozen for later experiments.

Conclusions: The project has the potential for a revolutionary impact on the treatment of breast cancer. There would be significantly fewer side effects than for conventional PDT, because the light-sensitive agent (SWNTs) is targeted specifically to the tumor. This therapy would be much less invasive than surgery and could replace surgery in some cases.

5. Neves, L.F.F., Lazrak, Y.A., Martyn, D.E., McFetridge, P.S., Resasco, D.E., and Harrison, R.G., "Novel Therapy for Cancer Using Single-Walled Carbon Nanotubes" Biomedical Engineering Society Annual Meeting, St. Louis, October, 2008.

To develop a therapeutic system with cancer cell selectivity, the present study proposes a possible specific and localized tumor treatment. Phosphatidylserine exposure on the external face of the cell membrane is almost completely exclusive to cancer cells and endothelial cells in the tumor vasculature. With this knowledge and because of the fact that the protein annexin V has excellent Ca^{2+}-phospholipid binding properties, we have excellent conditions for the development of a therapeutic system.

In this study, we have covalently coupled the human protein annexin V to a linker (fluorenylmethoxycarbonyl (Fmoc)-amine-PEG-succinimidyl carboxy methyl ester) adsorbed on single-walled carbon nanotubes (SWNTs). Human endothelial cells grown *in vitro* could be killed almost completely when SWNT-Fmoc-PEG-annexin V was bound to the cells and with laser light at 980 nm and an energy density of 195 J/cm^2. By contract, there was no harm to cells with SWNT-Fmoc-PEG-annexin V bound and no laser treatment,and there was no harm to cells receiving laser treatment and no SWNT-Fmoc-PEG-annexin V bound. The cell viability was evaluated by performing the Alamar Blue assay, cell counting and cell observation with a microscope.

6. Harrison, R.G. (speaker), Neves, L.F.F., Lazrak, Y.A., Martyn, D.E., McFetridge, P.S., Resasco, D.E., and Bartels, K.E., "Targeting Single-Walled Carbon Nanotubes for the Treatment of Cancer," AIChE Annual Meeting, Philadelphia, November, 2008.

Single-walled carbon nanotubes (SWNTs) are unique in that they strongly absorb near-infrared (NIR) light, while biological systems have very low levels of absorption of NIR light. This project focuses on developing a means for targeting the SWNTs to the tumor vasculature. It is proposed to target the SWNTs by conjugation with the human annexin V protein. Human annexin V (molecular weight of 36,000) is a monomeric protein that binds with high affinity to phophatidylserine (PS) in phospholipid bilayers. PS is the most abundant anionic phospholipid of the plasma membrane and is tightly segregated to the internal side of the plasma membrane in most cell types. Anionic phospholipids are largely absent from the external surfaces of resting mammalian cells under normal conditions. Recently, it has been found that PS is expressed on the external surface of endothelial cells that line the blood vessels in tumors but is not expressed on the outside surface of the vascular endothelium in normal organs. Thus, annexin V can be used to specifically target the endothelial cells of the tumor vasculature. The underlying hypothesis is that a SWNT-annexin V conjugate injected into the bloodstream will localize in the tumor vasculature and that exposure of the tumor to NIR radiation will cause the tumor to be selectively killed by a combination of heating and cutting off its blood supply.

In this study, we have covalently coupled the human protein annexin V to a linker (fluorenylmethoxycarbonyl (Fmoc)-amine-PEG-succinimidyl carboxy methyl ester) adsorbed on single-walled carbon nanotubes (SWNTs). Human endothelial cells grown *in vitro* could be killed almost completely when SWNT-Fmoc-PEG-annexin V was bound to the cells and with laser light at 980 nm and an energy density of 195 J/cm^2. By contract, there was no harm to cells with SWNT-Fmoc-PEG-annexin V bound and no laser treatment,and there was no harm to cells receiving laser treatment and no SWNT-Fmoc-PEG-annexin V bound. The cell viability was evaluated by performing the Alamar Blue assay, cell counting and cell observation with a microscope.

APPENDIX III

Personnel Receiving Pay from the Research Effort

Dr. Kenneth Bartels, Collaborator (Oklahoma State University)
Dr. Roger Harrison, Principal Investigator
Yahya Lazrak, Graduate Research Assistant
Luis Neves, Graduate Research Assistant
Brent Van Rite, Graduate Research Assistant